REMARKS

After entry of this amendment, claims 2, 4, 5 and 32-43 are pending. New claims 38-43 have been added and find support *inter alia* in the original claims. New claims 39 and 43 find further support in the specification, for example, at page 174, Table 1. New claim 40 finds further support in the specification, for example, at page 20, line 35, through page 21, line 6. New claim 41 finds further support in the specification, for example, at page 81, lines 13-18. The claims have been amended without prejudice or disclaimer to correct the antecedent basis and to address various points made in the Office Action, and find support *inter alia* in the original claims. No new matter has been added.

Claim Rejection – 35 U.S.C. § 112

Claims 2, 4, 5 and 33-37 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly lack of adequate written description support. Applicants respectfully disagree. However, to expedite prosecution, the claims have been amended without prejudice or disclaimer to recite the claimed subject matter with more specificity. It is noted that the claims as amended reflect a similar subject matter as recited in claim 32, which is not included in the present rejection. Accordingly, it is believed that the claims as amended overcome the rejection. Moreover, Applicants further believe that the written description requirement is satisfied for the reasons already of record in light of the present amendment.

For at least the above reasons and the reasons already of record, and further in light of the present amendment, reconsideration and withdrawal of the rejection is respectfully requested.

Claim Rejection – 35 U.S.C. § 102

Claims 35 and 37 are rejected under 35 U.S.C. § 102(b) as being anticipated by Qadota *et al.* (hereinafter "Qadota"). Applicants respectfully disagree and traverse the rejection. However, to expedite prosecution, the claims have been amended without prejudice or disclaimer to recite the claimed subject matter with more specificity. It is believed that the claims as amended overcome the rejection for the following reasons.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegall Bros., Inc. v.*

Union Oil Co., 814 F.2d 628, 631 (Fed. Cir. 1987). "[U]nless a reference discloses within the four corners of the document not only all the limitations claimed but also all of the limitations arranged or combined in the same way as recited in the claim, it cannot be said to prove prior invention of the thing claimed and, thus, cannot anticipate under 35 U.S.C. § 102." *Net MoneyIN Inc. v. VeriSign Inc.*, 545 F.3d 1359 (Fed. Cir. 2008).

The Examiner alleges that Qadota teaches a process of producing a fine chemical (i.e. the RAS2 protein) by introducing into a fungus (i.e. yeast) a nucleic acid molecule encoding SEQ ID NO: 2. The Examiner further asserts that Qadota shows that the introduction of the nucleic acid molecule confers an increase in the amount of the RAS2 protein in yeast. Characterizing yeast as a fungus, the Examiner concludes that Qadota anticipates claims 35 and 37. Applicants respectfully disagree with the Examiner's above assertions. Without acquiescing to the merits of the Examiner's argument, however, the claims have been amended without prejudice or disclaimer to specify that the organism useful for the claimed process is selected from the group consisting of bacteria, algae, non-human animals and plants. Since Qadota does not teach, expressly or inherently, introduction of a nucleic acid molecule encoding SEQ ID NO: 2 into an organism such as bacteria, algae, non-human animals or plants as recited in the claims as amended for the production of at least one fine chemical, Qadota does not teach all the claim limitations. Accordingly, Qadota does not anticipate the claims as amended.

For at least the above reasons and in view of the present amendment, reconsideration and withdrawal of the rejection is respectfully requested.

Claim Rejection – 35 U.S.C. § 103

Claims 2, 5, 32-34 and 36 are rejected under 35 U.S.C. § 103(a) as being obvious over Qadota in view of Sano *et al.* (hereinafter "Sano"). Applicants respectfully disagree.

The Examiner bears the initial burden of establishing *prima facie* obviousness. *See In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). To support a *prima facie* conclusion of obviousness, the prior art must disclose or suggest all the limitations of the claimed invention. *See In re Lowry*, 32 F.3d 1579, 1582, 32 USPQ2d 1031, 1034 (Fed. Cir. 1994).

Moreover, it is well established that under 35 U.S.C. § 103 the Examiner cannot selectively pick and choose from the disclosed parameters without proper motivation as to a particular selection. The mere fact that a reference may be modified to reflect features of the claimed invention does not make the modification, and hence the claimed invention, obvious unless the prior art suggested the desirability of such modification. *In re Mills*, 916 F.2d 680, 682, 16 USPQ2d 1430 (Fed. Cir. 1990); *In re Fritch*, 23 USPQ2d 1780 (Fed. Cir. 1992). "[A] patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art . . . it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements ***in the way the claimed new invention does***." *See KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1396 (2007) (emphasis added).

The Examiner relies on Qadota for allegedly teaching a process of producing a fine chemical (i.e. the RAS2 protein) by introducing into yeast a nucleic acid molecule encoding SEQ ID NO: 2. The Examiner acknowledges that Qadota does not teach the use of a plant to produce fine chemicals, but relies on Sano for such teaching. Specifically, the Examiner asserts that Sano teaches a tobacco plant transformed with a *rgp1* gene that overproduce salicylic acid ("SA," an organic acid), which was recovered (citing to Figures 1 and 3), and exhibit increased resistance to viral infection. Since the *rgp1* gene used in Sano encodes a Ras-related small GTP binding protein, the Examiner finds that one skilled in the art would have been motivated to transform another Ras-related small GTP binding protein coding gene such as RHO2 (i.e. SEQ ID NO: 1 encoding SEQ ID NO: 2) in order to increase the plant resistance to viral infection. The Examiner contends that one would have had a reasonable expectation of success given the positive result obtained with tobacco plants transformed with *rgp1*. The Examiner thus concludes that it would have been obvious for one of ordinary skill in the art to introduce a nucleic acid encoding SEQ ID NO: 2 into a plant. The Examiner additionally contends that it would have been obvious for one skilled artisan to recover SA because SA was known to be correlated with pathogen resistance and the method for its recovery was known in the art. Applicants respectfully disagree with the Examiner's above assertions and the finding of obviousness.

It is noted initially that the Examiner's above assertions in finding motivation and reasonable expectation of success is partially, if not wholly, based on an assumption that all Ras-related small GTP binding proteins are related to plant resistance to viral infection. Applicants strongly disagree.

As stated in Sano, little is known about the physiological functions of the small GTP binding proteins isolated from higher plants, while it is suggested that they are involved in diverse developmental processes. See Sano at page 10559, right Col., 1st full paragraph. These small GTP binding proteins can be classified into three major groups with distinct involvement in physiological processes and/or pathways: the Ras proteins, the Rho proteins, and the Rab/Ypt proteins. See Sano *et al.* (PNAS, 1995, 92: 4138-4144; copy attached; hereinafter "Sano-2") at page 4138, right Col., 1st full paragraph. The Ras proteins are involved in the transduction of external signals across the plasma membrane by interacting with Raf protein kinase, thus playing an intermediate role in phosphorylation cascade. The Rho proteins are critical for cytoskeletal organization by controlling cell division. The Rab/Ypt proteins function in intracellular transportation, or endocytosis and exocytosis, by activating individual vesicles around the endoplasmatic reticulum and golgi apparatus. *Id.* Thus, it is clear that the small GTP binding proteins classified within different group possess different physiological functions. A small GTP binding protein classified as being a Rho protein would therefore likely not function, and would likely not have been expected to function, as the same as another small GTP binding protein classified as being a Rab/Ypt protein, or *vice versa*.

It is noted that the *rgp1* gene used in Sano is a rab/ypt-related gene. See Sano at page 10556, left Col., 1st paragraph after Abstract. Thus, the protein encoded by the *rgp1* gene tested in Sano is a Rab/Ypt protein, which, as discussed above, would likely possess a very different physiological function than other small GTP binding proteins classified within the group of Ras proteins or Rho proteins. As such, one skilled in the art would not have been motivated to substitute the *rgp1* gene with a gene encoding either a Ras protein or a Rho protein, such as the RHO gene disclosed in Qadota, with a reasonable expectation of success that a similar positive result observed in Sano would be reproduced. Accordingly, Qadota and Sano is not combinable and for this reason alone, the rejection should be withdrawn.

Even assuming *arguendo* the references were combinable, it is respectfully submitted that the combined teaching does not teach or suggest all the limitations of the claimed subject matter and thus, does not render the claims *prima facie* obvious. As noted by the Examiner, Sano discloses tobacco transgenic plants overexpressing *rgp1*, a gene encoding a Ras-related small GTP binding protein. As described throughout the reference, the *rgp1* transgenic tobacco plants exhibit an increased level of SA production after wounding treatment. See e.g., Title at page 10556 ("induces salicylic acid in response to wounding") and page 10558, left Col. lines 3-9 (subtitle "Induction of SA by Wound Stress") and description of Figure 3 ("Induction of SA by would stress in the A2' plant"). Thus, it is clear that the increased production of SA in the *rgp1* transgenic tobacco plants observed in Sano is a result of wound stress rather than the overexpression of the *rgp1* gene. This is further evidenced in the data presented in Figure 3 at page 10558. As demonstrated in Figure 3, the production of SA significantly increases in the *rgp1* transgenic plants after wounding treatment as compared to the control plant (see e.g., 1, 2, 4, or 6 days after wound stress), while the level of SA has no significant difference between the control plants and the transgenic plants prior to the wounding treatment (see e.g., 0 day data). Accordingly, Sano does not teach or suggest that the overexpression of the *rgp1* gene in the transgenic tobacco plants confers an increase in the production of SA.

The combination of Sano with Qadota does not remedy this deficiency. As discussed above, Qadota is relied upon for its alleged teaching of a process of producing the RAS2 protein by introducing into yeast a nucleic acid molecule encoding SEQ ID NO: 2. Qadota does not teach or suggest any potential effect of introducing the disclosed RAS2 gene into a plant, let alone any possible effect in increasing the production of SA or any other fine chemicals in the plant. Accordingly, the combination of Qadota and Sano does not teach or suggest each and every limitation of the claimed subject matter and thus, does not render the claimed subject matter *prima facie* obvious. For this additional reason, the rejection should be withdrawn.

Because Qadota and Sano are not combinable, and because, even if combined, the combined teaching of the cited references does not teach or suggest each and every limitation of the claimed subject matter, a *prima facie* case of obviousness has not been established. For at least the above reasons, reconsideration and withdrawal of the rejection is respectfully requested.

Claim 4 is rejected under 35 U.S.C. § 103(a) as being obvious over Qadota in view of Sano, and further in view of Parker *et al.* (hereinafter "Parker"). Applicants respectfully disagree and traverse the rejection.

The above discussion concerning the teachings of Qadota and Sano is equally applicable here and thus, is explicitly incorporated by reference in its entirety.

It is noted initially that claim 4 is a dependent claim depending from claim 2. As discussed above, Qadota and Sano are not combinable, and even if combined, the combined teaching of Qadota and Sano does not teach or suggest each and every limitation of the subject matter claimed in claim 2. Parker is relied upon by the Examiner solely for the alleged teaching of a method for making and selecting plant mutants. Further, it is noted that Parker does not teach or suggest mutagenizing step as alleged by the Examiner since it is clearly stated that "[t]he mutation presumably arose from tissue culture-induced genetic variation, since the callus was not mutagenized prior to selection." See Parker at page 7178, right Col., 1st paragraph under "Discussion." Accordingly, the further combination of Parker with Qadota and Sano does not remedy the aforementioned deficiency found in the combined teaching of Qadota and Sano as applied to independent claim 2. Because Qadota and Sano do not render claim 2, the independent claim, obvious, the claim dependent therefrom, i.e. claim 4, is nonobvious. *See In re Fine*, 837 F.2d 1071, 1076 (Fed. Cir. 1988) (holding that if an independent claim is nonobvious then any claim dependent therefrom is nonobvious).

For at least the above reasons, reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

For at least the above reasons, Applicants respectfully request withdrawal of the rejections and allowance of the claims. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

Applicants reserve all rights to pursue the non-elected claims and subject matter in one or more divisional applications, if necessary.

Accompanying this response is a petition for a three-month extension of time to respond to the Office Action mailed November 10, 2009 with the required fee authorization. No further fee is believed due. However, if any additional fee is due, the Director is hereby authorized to charge our Deposit Account No. 03-2775, under Order No. 12810-00197-US from which the undersigned is authorized to draw.

Respectfully submitted,

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Attachment: Sano *et al.*, PNAS, 1995, 92: 4138-4144.

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Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Involvement of small GTP-binding proteins in defense signal-transduction pathways of higher plants

(cytokinins/DNA methylation/hypersensitive reaction/*rgp1*/wounding)

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ABSTRACT **Small GTP-binding proteins play a critical role in the regulation of a range of cellular processes—including growth, differentiation, and intracellular transportation. Previously, we isolated a gene, *rgp1*, encoding a small GTP-binding protein, by differential screening of a rice cDNA library with probe DNAs from rice tissues treated with or without 5-azacytidine, a powerful inhibitor of DNA methylation. To determine the physiological role of rgp1, the coding region was introduced into tobacco plants. Transformants, with *rgp1* in either sense or antisense orientations, showed distinct phenotypic changes with reduced apical dominance, dwarfism, and abnormal flower development. These abnormal phenotypes appeared to be associated with the higher levels of endogenous cytokinins that were 6-fold those of wild-type plants. In addition, the transgenic plants produced salicylic acid and salicylic acid-β-glucoside in an unusual response to wounding, thus conferring increased resistance to tobacco mosaic virus infection. In normal plants, the wound- and pathogen-induced signal-transduction pathways are considered to function independently. However, the wound induction of salicylic acid in the transgenic plants suggests that expression of *rgp1* somehow interfered with the normal signaling pathways and resulted in cross-signaling between these distinct transduction systems. The results imply that the defense signal-transduction system consists of a complicated and finely tuned network of several regulatory factors, including cytokinins, salicylic acid, and small GTP-binding proteins.**

As individual plants are unable to change their location, they have evolved numerous mechanisms that allow them to quickly respond to various environmental signals, including light, nutrients, and stresses—such as drought, pathogens, and wounding—and to adapt their behavior to the changing situation. To achieve the appropriate response, the external stimulus must be rapidly transformed to an internal signal that then triggers a whole series of biochemical and molecular pathways. This general concept postulates that external signals are transduced into chemical compounds and transmitted through a cascade of various proteins up to, for example, the DNA level. Among the numerous proteins that participate in this pathway, GTP-binding proteins play critical roles as signal transducers (1–5).

GTP-binding proteins, or G proteins, are equipped to bind GTP, which, because of the intrinsic GTPase activity of the proteins, can be hydrolized to GDP (3). This GTP/GDP inter-conversion is considered to enable the molecule to function as a "molecular switch," in which the GTP-bound form is active, and the GDP-bound form is inactive (4) and which can thus regulate the fundamental signaling pathways of organisms (5). Four major groups of G proteins have so far been distinguished: elongation factors, tubulins, membrane-bound trimeric G proteins, and small GTP-binding proteins. Among these, the latter two are known to be involved in signal transduction.

So far, >80 genes encoding small GTP-binding proteins have been documented from various organisms—including mammals, insects, yeasts, slime molds, and higher plants (6). The physiological functions of these proteins have not been fully clarified, but they have been classified into three major groups. The Ras proteins are involved in the transduction of external signals across the plasma membrane by interacting with Raf protein kinases, thus playing an intermediate role in the phosphorylation cascade (4, 5, 7). The Rho proteins are critical for cytoskeletal organization by controlling cell division (7, 8). The Rab/Ypt proteins function in intracellular transportation, or endocytosis and exocytosis, by activating individual vesicles around the endoplasmic reticulum and Golgi apparatus (7, 9). More than 30 individual genes encoding small GTP-binding proteins have been identified from higher plants—including *Arabidopsis*, pea, wheat, rice, maize, and tobacco (10). Most of them appear to belong to the Rab/Ypt subgroup, suggestive of some function in intracellular transportation, but little evidence has been provided (10). The *rha1* gene of *Arabidopsis* is predominantly expressed in developing guard cells, and Terryn *et al.* (11) conclude that it may function in vesicle transport. Expression of the *Pra2* and *Pra3* from pea, however, is down-regulated by light and mediated by phytochrome (12), suggestive of a role in developmental regulation.

We do not intend, however, to review the complex mechanisms of signal transduction here. Rather, we hope to assemble the results of our ongoing study of *rgp1*, a gene encoding a small GTP-binding protein, and to demonstrate how its gene product may be involved in the plant signal-transduction pathway.

Identification of *rgp1* Gene

Our study on *rgp1* began initially because of our interest in DNA methylation, and here we attempt to just briefly describe these findings in the hope of providing a basis for selecting *rgp1* for our further studies. In green plants, up to 30% of the total

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Abbreviations: BA, N^6-benzylaminopurine; JA, jasmonic acid; MeJA, jasmonic acid methylester; PI, proteinase inhibitor; PR, pathogenesis-related; SA, salicylic acid; TMV, tobacco mosaic virus; GTP[γS], guanosine 5'-[γ-thio]triphosphate.

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cytosine residues of genomic DNA consists of m^5C (13), which is thought to alter the higher conformation of DNA and consequently to change the interaction between DNA and DNA-binding proteins (14, 15). As such interactions may directly affect gene expression, our preliminary study determined whether plant growth was affected by its DNA-methylation status. Analysis of the relationship between DNA methylation and dwarfism in maize (*Zea mays*) showed that the amount of m^5C in the DNA of a single-gene dwarf mutant, d5, was ≈8% lower than that of its tall, near-isogenic counterpart (16). This relationship was further demonstrated in rice (*Oryza sativa*) seedlings treated with 5-azacytidine, a strong inhibitor of DNA methylation *in vivo* (17), in which both undermethylation and dwarfism induced by 5-azacytidine treatment were heritable. These results suggested that 5-azacytidine first induced demethylation of genomic DNA, thus causing an altered pattern of gene expression and consequently a reduction in plant height (18).

The next step in our studies was to identify gene(s) that had been affected by 5-azacytidine treatment and which, therefore, may have influenced the growth and development of the rice plants. A rice cDNA library was differentially screened with DNA probes of wild-type and 5-azacytidine-treated plants. Of the differentially expressed clones identified, one clone was the subject of further analysis (19). During growth of untreated control seedlings, expression of this clone was first observed 2 weeks after germination, reaching a maximum level between 4 and 6 weeks and gradually decreasing thereafter. In both 5-azacytidine-induced dwarf plants and their progenies, however, expression of the clone was markedly reduced throughout this entire period. As these results suggested that expression of the clone was influenced, either directly or indirectly, by DNA methylation and that the gene may be involved in some aspect of plant growth and development, the molecular structure of the gene was determined. The predicted protein encoded by the clone consisted of 226 amino acids with a relative molecular mass of 25 kDa. The putative amino acid sequence showed 62% identity to Ras-related Sp-ypt3, a small GTP-binding protein of fission yeast, whereas with other Ras-related proteins the identity ranged from 58% with ara, to 49% with Hrab1 and 45% with yeast YPT1. Such sequence similarities suggested that the gene belonged to the *ras*-related supergene family, and it was thus designated *rgp1*. Indeed, the *rgp1* gene product, expressed in *Escherichia coli*, was found to bind GTP (19).

Physiological Characteristics of Transgenic Tobacco Plants

Morphological Changes. To determine the physiological role of rgp1, the coding region of *rgp1* was introduced into tobacco plants in both sense and antisense orientations (20). Transformants, which contained the *rgp1* gene at one to three loci, showed distinct phenotypic changes, the most notable being a reduction in apical dominance with increased tillering, together with dwarfism, abnormal flower development, or both (Fig. 1). These morphological effects of the gene were observed in both sense and antisense transformants. Northern hybridization analysis showed that *rgp1* was expressed only in phenotypically abnormal transformants but not in apparently normal phenotypes. Furthermore, the R1 progenies from most transformants cosegregated in a 3:1 ratio for both kanamycin resistance and tillering, indicating that the observed abnormal morphology was indeed the result of the integrated *rgp1* gene. In addition, the expression of *tgp1*, a presumed tobacco homolog of *rgp1*, was markedly reduced in transformants expressing antisense *rgp1*, whereas it was apparently unaffected in transformants with the sense *rgp1*. These observations suggested that the phenotypic changes induced in antisense transformants may have been mediated by an effect on native *tgp1* mRNA, whereas, in the sense transformants, the changes may have been induced by overproduction of rgp1. This was, indeed, later confirmed by immunoelectrophoresis.

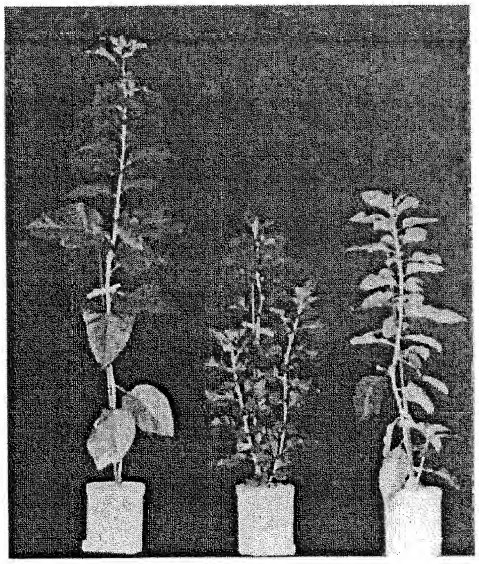

FIG. 1. Phenotypes of transformed and untransformed tobacco plants. Control, normal untransformed plant (left); transformed plant with sense-oriented *rgp1* gene (middle); and transformed plant with antisense-oriented *rgp1* gene (right).

Elevated Levels of Cytokinins. The involvement of cytokinins in the maintenance of apical dominance has been well documented (21, 22) and has also been directly demonstrated by the introduction of genes related to cytokinin biosynthesis into tobacco plants (23, 24). Other analyses have also shown that apical dominance is lost when the relative levels of auxins are decreased, suggesting that apical dominance is maintained by either the appropriate balance of auxin and cytokinin levels, the sensitivity of the plant tissues to them, or a combination of the two (25). The phenotypic changes in our transgenic plants suggested to us that the *rgp1* gene product may be involved at some point of either the hormone metabolic or response pathways.

To establish whether abnormal auxin or cytokinin levels were involved in the observed phenotypic effects, levels of zeatin and zeatin riboside, which are the major constituents of native cytokinins in higher plants, were measured in transgenic plants. Whereas the zeatin and zeatin riboside content in control plants was estimated at ≈5 pmol/g (fresh wt), that in transformants, with either sense or antisense oriented *rgp1*, increased to 23 pmol and 20 pmol/g (fresh wt), respectively (ref. 26 and unpublished data).

Abnormal Wound Response of Transgenic Plants

Induction of Acidic Pathogenesis-Related (PR) Proteins by Wounding. To identify native tobacco genes known to be affected by cytokinins in other plants and that show altered mRNA levels in the transgenic plants, Northern analysis was done with various cDNA probes by using total RNA from wild-type and several transgenic plants expressing the sense-oriented *rgp1* gene (26). Of the 14 various genes examined, only those encoding acidic PR proteins (PR-1, PR-Q, and PR-S) showed elevated transcript levels in the transgenic plants. However, the accumulation of the PR-1 proteins was not constitutive but rather occurred only in response to wounding. Thus, when a leaf was wounded by punching out discs every 2 days, PR-1 proteins accumulated up to 600 μg/g (fresh wt) by the 10th day. In addition, when a transgenic plant

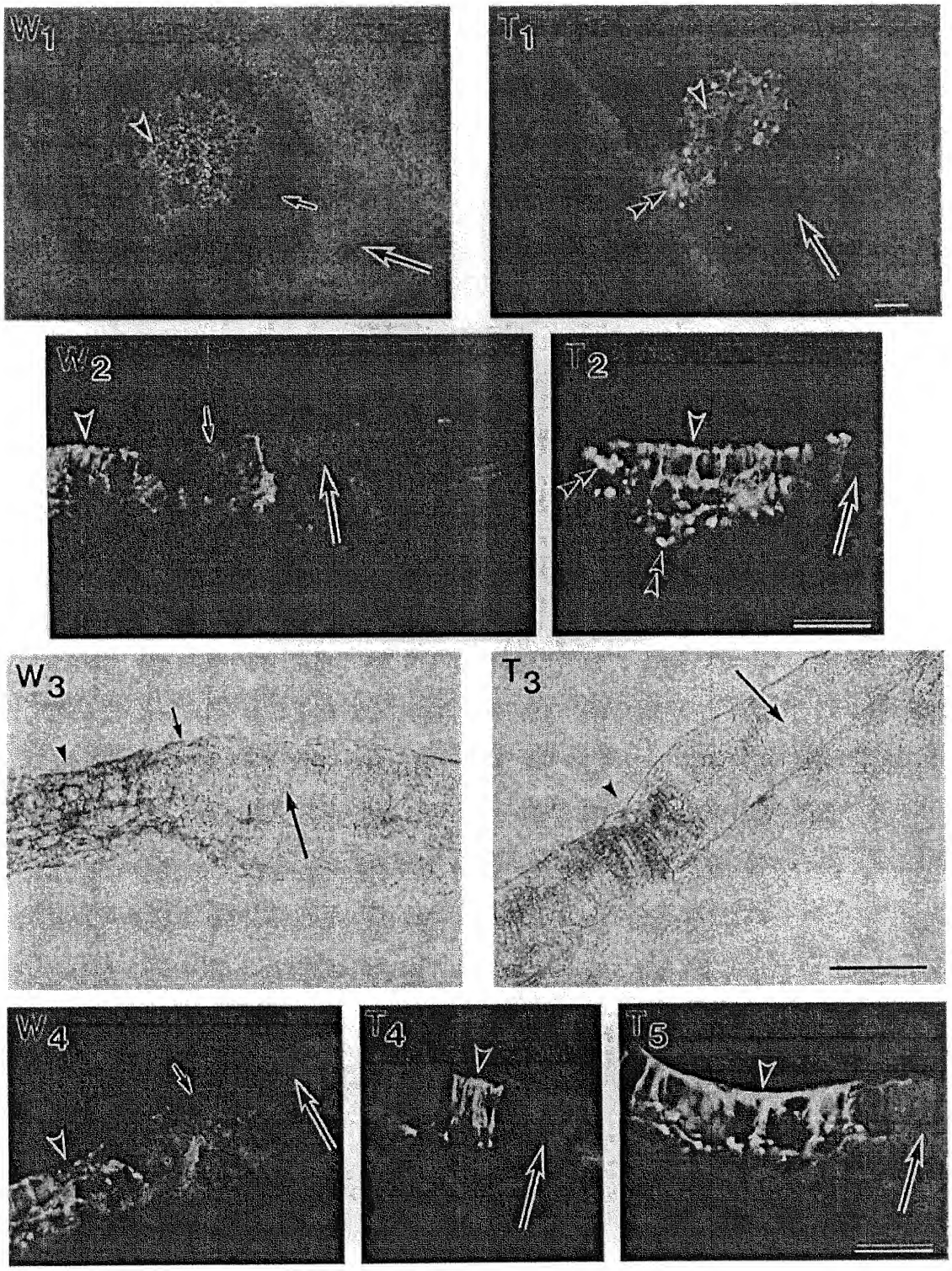

FIG. 2. Enhanced hypersensitive reaction of transgenic tobacco with sense-oriented *rgp1*. Detached leaves were inoculated with TMV and incubated at 20°C under continuous light for the appropriate time interval. Discs containing local lesions were punched out, stained with aniline blue, and observed under UV epifluorescence. The center of the necrotic lesions is indicated by arrowheads, callose by double arrowheads, browning by small arrows, and healthy regions by large arrows. Whole local lesions, 6 days after inoculation, are shown for wild-type (W_1) and transgenic (T_1) plants, and the corresponding cross-sections are also shown (W_2, T_2, respectively). Cross-sections, prepared 36 hr after inoculation, were cleared with ethanol to observe necrotic lesions (W_3 and T_3). Cross-sections, prepared 6 days after inoculation, and observed by autofluorescence, show browning and healthy chlorophyll (W_4, T_4, and T_5). Note that while chlorophyll decay begins at the edge of the lesion in wild-type plants (small arrow), indicative of rapid spread of virus attack, the decay is limited to a small area in transgenic plants. (Bars = 200 μm.)

was wounded by detaching lower leaves, the upper unwounded leaves also initiated PR-1 protein production up to 150 μg/g (fresh wt) by the eighth day, indicating that PR-1 protein induction was systemic. In contrast, transcript levels of the PI-II gene, which is normally induced by wounding, were generally suppressed in the same wounded plants.

PR proteins were originally identified in hypersensitive tobacco plants carrying the *N* gene upon viral infection (27). Although the molecular functions of PR proteins are not fully clarified, they apparently are important in the plant self-defense system (28). Indeed, some PR proteins exhibit antifungal activity against certain phytopathological fungi (29–31). The PR proteins consist of both acidic and basic isoforms that differ in their tissue and organ localizations and in the signals by which they are induced (28, 30, 32, 33). The acidic PR proteins are induced by the hypersensitive reaction after tobacco mosaic virus (TMV) infection or salicylic acid (SA) treatment and, to a lesser extent, by cytokinins (32) and auxins (34), whereas the basic PR proteins are efficiently induced by wounding or ethephon (ethylene) treatment as well as TMV infection (32, 33) and are actually inhibited by auxins and cytokinins (35). Production of a large amount of acidic PR proteins in response to wounding is thus an abnormal response of the transgenic plants.

Induction of SA and Salicylic Acid-β-Glucoside by Wounding. As SA is a powerful inducer of acidic PR proteins, the question arose whether or not SA was involved in the abnormal wound induction of PR proteins in the transgenic plants. We therefore examined SA levels in young leaves after injuring the upper epidermis of leaves with carborundum (26). Samples, taken after appropriate intervals, were used to quantify endogenous SA levels. The leaves began producing SA 1 day after injury to a maximum of 180 ng/g (fresh wt) from the second day for at least an additional 6 days. In wounded transgenic plants, salicylic acid-β-glucoside was more rapidly synthesized than SA so that its level exceeded 700 ng/g (fresh wt) from before 6 hr after injury up to at least 2 days. In addition to the wounded leaves, however, unwounded adjacent leaves also produced SA and salicylic acid-β-glucoside, although their levels were ~30% those of the injured leaves, indicating that the wound response of transgenic plants was systemically transmitted to the neighboring leaves. We therefore concluded that the induction of SA and salicylic acid-β-glucoside by wounding constituted the abnormal response of the transgenic plants.

Enhanced Hypersensitive Reaction by TMV Infection. As exogenously applied SA can suppress TMV multiplication without affecting sensitivity to the virus (36), we tested the susceptibility of the transgenic plants to TMV infection at 20°C. Necrotic lesions in the transgenic plants were visible within 24 hr of inoculation (26), suggesting that these plants have an enhanced hypersensitive response to pathogens in comparison with control plants, which require at least 36 hr to develop visible local lesions. Six days after inoculation of the detached leaves with TMV, the average size of necrotic lesions in the wild-type and transgenic plants was 2.10 mm and 0.34 mm, respectively. Although the number of local lesions was almost the same, the amount of TMV recovered from the same number of lesions was >12-fold higher in wild-type plants than in transgenic plants (data not shown).

Resistance of the transgenic plants was further analyzed by histochemical assays (Fig. 2). TMV-infected tobacco plants are found to have callose (β-1,3-glucan) deposited in and around necrotic lesions, and this deposition is generally regarded as proportional to the increase in systemic acquired resistance (37–39). Therefore, we first examined callose deposition as a marker for the resistance of our transgenic plants. Six days after TMV inoculation, the transgenic plants developed heavy callose deposition, as indicated by the intense bright-yellow fluorescence (Fig. 2, T_1 and T_2), whereas wild-type plants showed almost no such deposition (W_1, W_2). The size of brown rings, outer regions of the necrotic lesions where TMV actively multiplies, was large in the wild type plant and small or missing in the transgenic plants. This result was confirmed by cytochemical observations, in which the necrotic cells spread within 36 hr of inoculation from the upper to lower epidermis of wild-type plants (Fig. 2, W_3), whereas necrotic regions were limited to a small portion of the upper epidermis and palisade cells of the transgenic plants (Fig. 2, T_3). This situation was maintained in both wild-type and transgenic plants for at least 6 days after inoculation, as shown by an autofluorescence assay (Fig. 2, W_4, T_4, and T_5). These observations suggest that hypersensitive cell death occurred quickly in the transgenic plants and resulted in an enhanced hypersensitive response to prevent further viral multiplication.

Signal Crossing of Wound- and Pathogen-Signaling Pathways in Transgenic Plants

Due to its diverse biological functions, such as systemic induction of PR proteins, ion uptake, stomatal closure, and flowering of thermogenic plants, SA was suggested to function as a native regulator and even possibly as a phytohormone (40). In particular, SA is considered to be involved in various signal-transduction systems, especially those leading to systemic acquired resistance against pathogen attack (41, 42), because induced or increased SA levels followed by subsequent increases in acidic PR proteins are seen in tobacco and cucumber plants upon infection with TMV (43) or the fungal pathogen *Colletotrichum* (44), respectively. In marked contrast, SA is not induced in response to wounding (43). Thus, transduction of the wound signal is thought to follow a separate pathway from that of SA (41) and to involve jasmonic acid (JA), which is synthesized through a lipid-based signal-transduction system (45). In turn, JA may act as a direct intracellular signal intermediate and effect a subsequent change in the expression of specific sets of genes, such as those encoding proteinase inhibitors (PIs) (46, 47). In normal plants, therefore, the signal cascade involving SA is regulated quite distinctly from that involving JA and its methyl ester (MeJA).

In the transgenic plants expressing *rgp1*, however, the two normally distinct pathogen and wound signal-transduction systems appear to be modified, possibly by an unusual crossing of the signals, with the result of abnormal SA production in response to wounding. Although this disorder is clearly due to the integrated *rgp1* gene, questions arise about the mechanism by which rgp1 modifies these signal pathways. In an attempt to address these questions, we examined, in transgenic plants, the differential expression of PR-1 and PI-II transcripts that are normally induced by SA and JA (MeJA), respectively, in wild-type plants (Fig. 3). Healthy intact leaves of both wild-type and transgenic plants produced neither of these transcripts, whereas wounding induced accumulation of PI-II transcripts in both sets of plants. In addition, however, wounding induced a large accumulation of PR-1 transcripts in transgenic plants and a trace amount in control plants. MeJA enhanced the accumulation of PI-II transcripts in both plants but severely reduced the PR-1 transcript levels induced by wounding in the wild-type plant and especially in the transgenic plant. In contrast, SA further increased transcript levels of PR-1 induced by wounding but reduced transcript levels for PI-II in both plants. N^6-Benzylaminopurine (BA) also enhanced PR-1 transcript levels induced in both plants by wounding but either suppressed or had no affect on the PI-II transcripts in wild-type and transgenic plants, respectively. Guanosine 5′-[γ-thio]triphosphate (GTP[γS]), a powerful inhibitor of G proteins *in vivo* (48), suppressed wound-induced PR-1 and PI-II transcript accumulation in the wild-type plant, whereas it was apparently ineffective in the transgenic plant.

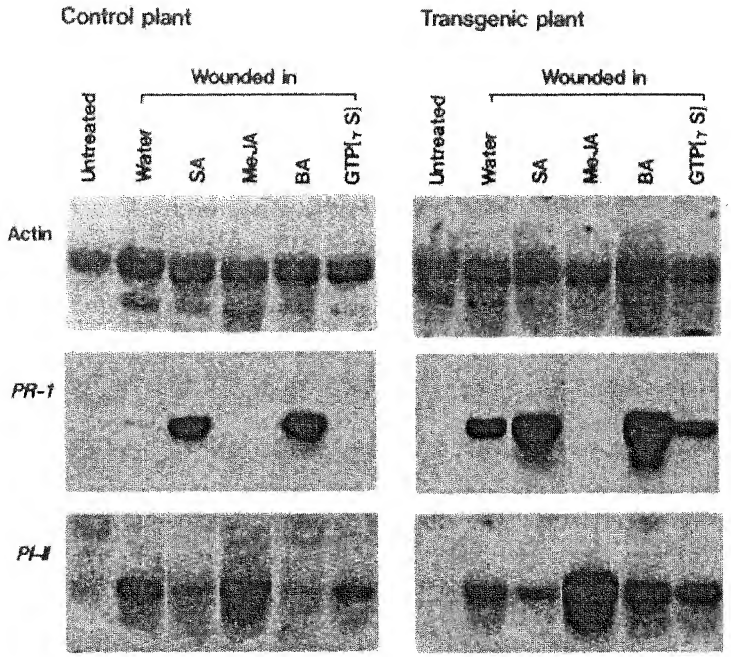

FIG. 3. RNA hybridization analysis in wild-type and transgenic tobacco plants. A whole leaf from either a wild-type or transgenic plant with the sense-oriented *rgp1* was detached, cut into pieces, and floated on water or on a solution containing either 50 μM SA, 50 μM MeJA, 20 μM BA, or 25 μM GTP[γS] at 20°C under continuous light for 40 hr and then used for total RNA isolation. As a control, total RNA was immediately isolated from untreated, freshly harvested leaves (untreated). A 30-μg RNA aliquot was assayed for mRNA levels of actin, as an internal standard, and PR-1 and PI-II by Northern blot hybridization.

These observations clearly suggested that G proteins are indeed involved in the PR-1 and PI-II induction pathways. Because the integrated *rgp1* gene appears to overcome the inhibitory effects of GTP[γS], we consider that small GTP-binding proteins, homologous to rgp1, are probably involved in these pathways. The results also show that JA and SA function antagonistically in the induction of PR-1 and PI-II transcripts. Indeed, reduction in PR-1 transcript levels is directly associated with reduced levels of SA and salicylic acid-β-glucoside after JA treatment of the transgenic plants (data not shown). Therefore, JA could inhibit SA biosynthesis. Overall, our data imply that small GTP-binding proteins, cytokinins, JA, and SA are equally necessary for correct functioning of the defense response.

Protein Transportation. Because rgp1 belongs to the Rab/Ypt subgroup of small GTP-binding proteins, thought to be involved in intracellular transportation, the question arose whether or not the abnormal signal crossing was due to problems in protein transport. An initial immunoblot assay showed that most rgp1 proteins in transgenic plants were located in the microsomal fraction, suggestive of some role in transportation (data not shown). Subsequently, we examined the transport of acidic PR-1 proteins, which are normally secreted into intercellular spaces, as a representative protein. Results from both intact leaves and suspension-culture cells showed that the proteins were normally transported (26), suggesting that the observed disturbance in the wound signals was not the result of a disturbance in PR protein transportation, although the transport of other proteins involved in the signal pathway could have been modified.

Are Cytokinins Involved in the Defense Response? As (*i*) it is generally established that some phytohormones, especially cytokinins, auxins, ethylene, and abscisic acid, are involved in the pathogen- and wound-signaling pathways (28, 49) and as (*ii*) our transgenic plants constitutively produce high levels of cytokinins even without wounding (data not shown), the hormonal imbalance induced by rgp1 could be the primary cause of the observed effects. This hypothesis is corroborated by our findings that, in transgenic plants, PR-1 mRNA accumulation in response to wounding is completely suppressed by exogenous application of a cytokinin antagonist, 2-chloro-4-cyclohexylamino-6-ethylamino-*s*-triazine (data not shown), indicating that cytokinins are a prerequisite for *PR-1* expression. Furthermore, the transgenic plants are insensitive to exogenously applied BA to suppress *PI-II* (Fig. 3), suggestive of some sort of disturbance in the cytokinin and JA signaling pathways. However, when we compared the wound response between transgenic plants containing *rgp1* with those containing *ipt*, which constitutively produce endogenous cytokinins, we found that although the *ipt* transformants accumulated a 5-fold higher level of SA after a single wound in comparison with wild-type plants, these plants were not particularly resistant to virus infection. This result is consistent with the observation that, in TMV-inoculated leaf pieces of wild-type tobacco, exogenously applied cytokinins could not suppress viral multiplication (unpublished observation). These results suggest that cytokinins are essential, but not sufficient, for induction of the defense response. It is, therefore, attractive to speculate that cytokinins, through the action of related GTP-binding proteins, regulate the JA and SA levels so that in normal plants the external signals are correctly transmitted to the relevant transduction pathways.

Concluding Remarks—The Defense Signal Pathway Network

On the basis of the above observations, we currently propose a two-step mechanism to explain the abnormal behavior of our transgenic plants: (*i*) small GTP-binding proteins play an essential role in cytokinin biosynthesis and/or metabolism; and (*ii*) small GTP-binding proteins are also directly involved in the defense signaling pathways. Although we are unclear about the molecular mechanisms involved, we are certain that introduction of the *rgp1* gene into the host plant seriously disturbs cytokinin biosynthesis and/or metabolism, resulting in its accumulation in leaves. Such abnormally high cytokinin levels may pleiotropically affect growth and development and also possibly the defense signal pathways by, for example, sensitizing the wound-perception system. Also, the presence of excess rgp1 in sense plants or the absence of its native tobacco homologs in antisense plants may disturb the normal signaling pathways that are mediated by numerous different small

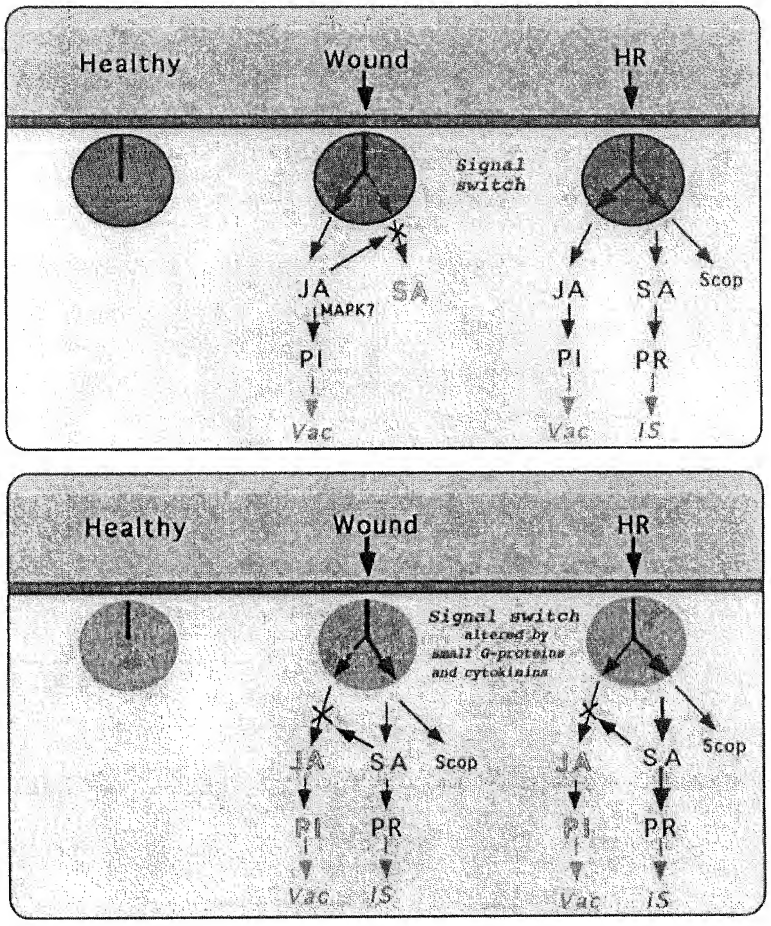

FIG. 4. Tentative signal flows of wound and hypersensitive reaction (HR) after pathogen attack. (*Upper*) Wild-type tobacco plant. (*Lower*) Transgenic (*rgp1*) tobacco plant. Activated signals are indicated by solid letters, and suppressed signals are indicated by open letters. The signal channel involving ethylene is omitted to simplify the illustration. Is, intercellular spaces; PI, PI-II; PR, acidic PR proteins; Scop, scopoletin; Vac, vacuole; MAPK, mitogen-activated protein kinase.

GTP-binding proteins. In fact, we have observed several abnormal biochemical responses in transgenic plants, probably due to such disturbed or altered signals. For example, upon wounding, transgenic plants produced high levels of scopoletin, a derivative of coumarins, which has strong antimicrobial activity and which in wild-type plants is not induced by wounding but by the hypersensitive reaction (50). Furthermore, the activity of phenylalanine ammonia-lyase, an enzyme involved in the first step of the biosynthesis of SA and coumarin derivatives (51), is continuously increased after wounding of transgenic plants, whereas this enzyme is only transiently increased in wounded wild-type plants.

It is therefore highly probable that the defense signal pathways consist of a complicated, yet well-regulated, network of several regulatory factors including cytokinins, SA, JA, and GTP-binding proteins. In addition, we have recently identified a gene encoding a mitogen-activated protein kinase homolog, which shows increased transcript levels after exogenous application of MeJA (data not shown). Thus protein kinases also appear to be involved in these signaling pathways. A tentative model for the wound and HR signal flows, incorporating these present and previous findings, is illustrated in Fig. 4. In this model, we introduce the concept of a "signal switch," which directs the input signals to the appropriate transduction channels under the control of cytokinins and small GTP-binding proteins. In wild-type plants, the wound signal is directed to the JA channel, and the HR signal is directed to both the JA and SA channels, whereas in transgenic plants both signals are preferentially directed to the SA channel.

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